Branchial expression of COX-2, nNOS, and transporters following rapid transfer of *Fundulus heteroclitus* from fresh water to seawater

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Killifish can tolerate acute transfer from fresh water to seawater with only minor, transient elevations in blood plasma osmolarity and Na^+ concentration[5] by rapidly stimulating NaCl secretion[6], and presumably inhibiting ion absorption. Although the key ion transporters involved in NaCl secretion have been characterized and candidate transporters for ion absorption have been identified[3], little is known about transcriptional regulation of these transporters immediately following acute salinity transfers. Transcriptional and post-transcriptional regulation of these transporters is thought to be at least partially mediated by paracrine signaling pathways[2]. For example, we previously used pharmacological inhibitors to demonstrate that prostanoids and nitric oxide (NO) inhibit short circuit currents in the killifish opercular membrane (a model for salt secretion in teleost gills), suggesting that cyclooxygenase (COX) and nitric oxide synthase (NOS) regulate NaCl transport in teleost gills[4]. In this study, we measured a time course of mRNA expression changes for COX2, nNOS, and several transporters for 24 hours following acute transfer from fresh water to seawater to determine if, and how, transcription of these paracrine agents and transporters is altered to stimulate NaCl secretion and inhibit NaCl absorption in the gills of teleosts.

Twenty killifish were shipped from the Mount Desert Island Biological Laboratory (MDIBL) to the University of Florida and held in a 380 l tank containing buffered Gainesville tap water (fresh water: conductivity ~1400 µs, pH ~8.2, 20°C) for 37 days. Fifteen killifish were then transferred directly to another 380 l tank containing buffered seawater (conductivity ~47 ms, pH ~8.2, 20°C). After 3, 8, and 24 hours of exposure to seawater, killifish were removed and their gills were frozen; the five killifish that remained in fresh water were used as controls. Poly A RNA was reverse transcribed from total RNA as described previously[1] and the resulting cDNA was subjected to PCR in the presence of SYBR® Green (Molecular Probes, Inc., Eugene Oregon) binding dye at MDIBL. All PCR reactions were run in triplicate and included 0.2 ul of cDNA (2.0 ul of a 1/10 dilution of original cDNA), 7.4 pmoles of each primer, and SYBR® Green Master Mix (Applied Biosystems, Foster City, CA) in a total volume of 25 ul. Primers were designed from *Fundulus* specific cDNA sequences that we either cloned using degenerate primers (L8, COX2:AY532639, nNOS:AY533030, NHE3:AY818825, NHE2, and V-ATPase) or derived from GenBank (CFTR:AF000271, NKCC:AY533706, and NKA1:AY057072). All real-time PCR reactions were run in a Stratagene MX4000 Real-Time Quantitative PCR system (Stratagene, La Jolla, CA) with standard cycling parameters, and melting curve analysis was used to verify the amplification of a single product in each well. Relative gene expression was calculated from a relative standard curve that used serial dilutions of a pooled gill cDNA sample as the template, and all results were normalized to ribosomal protein L8, a highly conserved gene for which expression in the gills remains constant during salinity changes (unpublished observation). ANOVAs with Dunnett's post hoc tests were used to compare seawater to freshwater control expression levels.

Interestingly, expression of the three NaCl secretion transporters increased with variable kinetics and magnitudes. For example, NKCC and NKA1 expression increased only moderately after 24 hours, but CFTR expression was elevated as early as three hours, reaching a maximum of 2.8 fold at eight

hours (Table 1). Our results confirm a previous study that observed rapid increases in CFTR expression following transfer from fresh water to seawater[7], and suggest that the apical Cl^- channel may be a rate-limiting step in the NaCl secretory mechanism. Expression of the basolateral NKCC and NKA1 transporters may be constitutively high enough so that rapid transcription is not required. Of the three Na^+ and/or acid transporters that could potentially have a role in active Na^+ absorption, only NHE3 had a significant decrease in expression following transfer to seawater (Table 1). This is one of the first demonstrations that salinity influences NHE expression in the gills of teleosts, and suggests that NHE3 may be responsible for Na^+ absorption. COX2 expression had the largest change in expression for any mRNA with a transient, 3.4-fold increase three hours after transfer to seawater (Table 1). Alternatively, nNOS expression did not change and had the latest threshold cycle numbers, suggesting that its expression levels are low (unpublished observation). These data agree with our previous pharmacological evidence that prostanoids have a larger quantitative role in controlling NaCl transport in killifish opercular membranes than NO[4]. Further work is being done to localize COX2 and NHE3 protein in the gills and to determine the effects of transfer from seawater the fresh water on the same transcripts.

Table 1. Relative expression of putative paracrine and ion transport enzymes in the gills of killifish following acute transfer from fresh water (FW) to seawater. Each cDNA was quantified by real-time RT-PCR and normalized to ribosomal protein L8. N=5, * $p < 0.05$.

mRNA	FW	3 h	8 h	24 h
COX2	1.00 ±0.14	3.38 ±0.61*	1.39 ±0.28	1.04 ±0.20
nNOS	1.00 ±0.60	1.04 ±0.93	0.65 ±0.54	0.23 ±0.18
NKCC	1.00 ±0.13	1.20 ±0.16	1.07 ±0.16	1.87 ±0.27*
CFTR	1.00 ±0.13	1.99 ±0.28	2.77 ±0.41*	1.98 ±0.43
NKA1	1.00 ±0.09	1.07 ±0.10	1.04 ±0.12	1.38 ±0.05*
NHE3	1.00 ±0.08	0.98 ±0.05	0.49 ±0.10*	0.49 ±0.09*
NHE2	1.00 ±0.32	0.74 ±0.15	1.35 ±0.51	3.26 ±1.93
V-ATPase	1.00 ±0.09	0.98 ±0.07	0.78 ±0.08	0.89 ±0.08

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1. **Choe, K.P., J.W. Verlander, C.S. Wingo, and D.H. Evans**, A putative H^+/K^+-ATPase in the Atlantic stingray, *Dasyatis sabina*: primary sequence and expression in gills. *Am. J. Physiol.*, 287: R981-R991, 2004.
2. **Evans, D.H.**, Cell signaling and ion transport across the fish gill epithelium. *J. Exp. Zool.*, 293: 336-347, 2002.
3. **Evans, D.H., P.M. Piermarini, and K.P. Choe**, The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. *Physiol. Rev.*, 85: 97-177, 2005.
4. **Evans, D.H., R.E. Rose, J.M. Roeser, and J.D. Stidham**, NaCl transport across the opercular epithelium of *Fundulus heteroclitus* is inhibited by an endothelin to NO, superoxide, and prostanoid signaling axis. *Am. J. Physiol.*, 286: R560-568, 2004.
5. **Jacob, W.F. and M.H. Taylor**, The time course of seawater acclimation in *Fundulus heteroclitus* L. *J. Exp. Zool.*, 228: 33-39, 1983.
6. **Marshall, W.S., T.R. Emberley, T.D. Singer, S.E. Bryson, and S.D. McCormick**, Time course of salinity adaptation in a strongly euryhaline estuarine teleost, *Fundulus heteroclitus*: A multivariable approach. *J. Exp. Biol.*, 202: 1535-1544, 1999.
7. **Singer, T.D., T. Stephen J, W.S. Marshall, and C.F. Higgins**, A divergent CFTR homologue: highly regulated salt transport in the euryhaline teleost *F. heteroclitus*. *Am. J. Physiol.*, 274: C715-C723, 1998.